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ORIGINAL PAPER

# Soil microbial mechanisms of *Stevia rebaudiana* (Bertoni) residue returning increasing crop yield and quality

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**Abstract** Due to the ever-increasing worldwide plantation of sweet leaf *Stevia rebaudiana* (Bertoni), how to efficiently and effectively utilize the huge amounts of leaf residues that contain abundant nutrients after sweetener extraction becomes an eminent issue. One option is to return these residues into soil, as organic manure in the fresh or composted form, in order to both sustain soil fertility and avoid potential environmental pollution. In a field experiment, we studied if the *Stevia* leaf residue returning affected both plant and soil microbial growths as well as the possible change of soil microbial community composition. In doing so, four treatments were employed: (1) no chemical fertilization and no *Stevia* residue returning (no-fertilization control); (2) chemical N, P, and K fertilization (NPK); (3) fresh *Stevia* residue plus NPK (FS + NPK); and (4) composted *Stevia* residue plus NPK (CS + NPK). Responses of plant and soil microbial communities to *Stevia* residue input after 1-year fertilizations were investigated by multiple approaches, including soil enzyme assay, real-time quantitative polymerase chain reaction, and PCR–denaturing gradient gel electrophoresis. Our results showed that compared to the sole NPK and no-fertilization control, returning *Stevia* residues to soil stimulated the enzyme activities of dehydrogenase, invertase, and urease, except neutral phosphomonoesterase; thereby, both the *Stevia* leaf biomass and sweet glycoside of rebaudioside A were increased. The soil microbial community abundance was increased by the returning of *Stevia* residues, and their composition was shifted, evidenced by an increase of relative abundance of some genotypic groups, such as *Bacillales*. Further molecular analysis of *Bacillus* confirmed that this guild composition was positively influenced by *Stevia* residue returning, especially for *Bacillaceae*. Our results demonstrated an effective use of *Stevia* residues as organic manure for promoting *Stevia* yield and quality through stimulating soil microbial growth and enzyme activities.

**Keywords** *Bacillus* · Dehydrogenase · Invertase · Neutral phosphomonoesterase · Urease · Soil microbial community

## Introduction

The species *Stevia rebaudiana* (Bertoni) is a perennial herb belonging to the Asteraceae family. Its leaf contains natural low-calorie sweeteners, primarily as entkaurine stevioside and rebaudioside A, which are 300 times sweeter than sucrose at the same concentration (Bondarev et al. 2002). These sweeteners can cure hypotensive, heart tonic actions and serve as non-cariogenic sucrose substitute for patients suffering from obesity and dental maladies (Ferri et al. 2006; Geuns 2003; Puri et al. 2011). These unique properties make *Stevia* in huge demand in pharmaceutical, food, and beverage industries and further lead to the urgent demand for the large-scale *Stevia* plantation, particularly in the recently rapid economic growth in China (Puri et al. 2011). Although studies on increases in *Stevia* production and its natural sweetness have been conducted (Kothari et al. 2009), information on how soil properties affect *Stevia* plant growth, yield, and quality is limited.

Soil microorganisms are key constituents of soil ecosystems, and their positive responses are in favor of the increase

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in nutrient cycling and soil fertility in a sustainable and economic way (Lueders et al. 2006). Long-term experiments showed that fertilization with organic matter can help promote crop growth and quality (Cai and Qin 2006). The possible underlying mechanisms suggest that both soil microbial growth and diversity are enhanced (He et al. 2008), whilst soil microbial community structure is shifted (Börjesson et al. 2011). However, information is limited on how the return of *Stevia* residues could affect soil properties, especially soil microbial activities under continuous *Stevia* plantation.

Meanwhile, *Stevia* residues after sweetener extraction, a water extractable process without chemical or ethanol (Puri et al. 2011), still contain a variety of minerals and small molecular weight organic acids (Lemus-Mondaca et al. 2012; Wölwer-Rieck 2012). These materials are ideal energenic C and nutritional sources for promoting soil microbial growth, although *Stevia* extract is found to inhibit some bacterial growth by pour plate method (Debnath 2008), due to the existence of flavonoids (Geuns 2003) and phenolic compounds (Kim et al. 2011). However, so far, no reports have studied the disposal of *Stevia* residues after the extraction of sweeteners, whilst a large-scale industrialization of *Stevia* inevitably produces a large amount of *Stevia* residues. For instance, only a small part of *Stevia* residues are used to feed animals, and the majority of them, as resource waste and pollution, are risks to the environment in China. A rational returning of *Stevia* residues to the soil may provide efficient pathways of utilizing waste resources as renewable manure for benefiting both industrial and agricultural sectors.

The aim of this study was therefore to evaluate the effects of returning *Stevia* residues as organic materials on the yield and quality of *Stevia* itself and the potential responses of soil microorganisms to such a residue addition. As a result, two kinds of *Stevia* leaf residues, the composted and fresh, were used as organic manure to compare their field practice under the current chemical fertilization. In doing so, four fertilization treatments were employed: (1) no chemical fertilization and no *Stevia* residue retuning control (no-fertilization control); (2) chemical N, P, and K fertilization (NPK); (3) fresh *Stevia* residue plus NPK (FS + NPK); and (4) composted *Stevia* residue plus NPK (CS + NPK). *Stevia* yield and quality were measured, and soil microbial functions, abundances, and community compositions were analyzed by soil enzyme assays, real-time quantitative polymerase chain reaction (qPCR), and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) approaches. This study will provide useful information on increasing *Stevia* yield and quality and the changing of soil microbial communities by a sustainable and economic pathway of utilizing *Stevia* residues.

## Materials and methods

### Site description and plant material

A field experiment was carried out at Zhucheng (36°40′N, 119°34′E, ~61 m above sea level), Shandong, China. This region has an average annual precipitation of 750 mm and a mean annual temperature of 12 °C. The main soil characteristics in the 0–15-cm surface layer were: 12.8 $g kg^{-1}$ of organic C, 7.4 $g kg^{-1}$ of total N, 10.3 $mg kg^{-1}$ of mineral N ($NH_4^+$-N and $NO_3^-$-N), 37.9 $mg kg^{-1}$ of available P, 365.0 $mg kg^{-1}$ of available K, and pH 7.40 (soil/water = 1:2.5).

*S. rebaudiana* (Bertoni) had been cultivated for 3 years with conventional management of chemical fertilizer. The *Stevia* residues, mainly leaves, produced after extracting the stevioside from *Stevia*, were used in experiments. The characteristics of fresh *Stevia* residue were: 21.9 $mg g^{-1}$ of total N, 1.1 $mg g^{-1}$ of total P, and 1.2 $mg g^{-1}$ of total K. The properties of the composted residues (naturally matured over half a year without adding other materials) were: 42.0 $mg g^{-1}$ of total N, 1.5 $mg g^{-1}$ of total P, and 3.2 $mg g^{-1}$ of total K.

### Experiment design

To evaluate the effects of the returning of *Stevia* residue to soil on *Stevia* growth and soil microbial properties, four fertilization treatments with three replicates each were arranged in a randomized block design. These four treatments were: (1) no chemical fertilization and no *Stevia* residue retuning control (no-fertilization control); (2) chemical N, P, and K fertilization (NPK); (3) fresh *Stevia* residue plus NPK (FS + NPK); and (4) composted *Stevia* residue plus NPK (CS + NPK). The fertilization rates were 108 $kg N ha^{-1}$ (urea), 60 $kg P_2O_5 ha^{-1}$ (super phosphate), and 130 $kg K_2O ha^{-1}$ (potassium sulfate) under the NPK treatment. The same amounts of N, P, and K were used under the FS + NPK and CS + NPK treatments by considering the respective content in the *Stevia* residues. Both P fertilizer and residues were applied as basal fertilization, whereas N and K fertilizers were applied two times (60 % as basal and 40 % as supplementary fertilization).

### Soil sampling

After 2 months of supplementary fertilization, soils (0–20 cm) were sampled on 20 August 2011 after *Stevia* harvest. Soils from six points of each replicate were collected by an auger, sieved (<2 mm), and mixed as one composite sample after the removal of debris and plant materials. Soil samples for molecular and chemical determinations were kept at −20 and 4 °C, respectively.

### Determination of soil dehydrogenase, neutral phosphomonoesterase, invertase, and urease activity

Soil dehydrogenase activity was determined by the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to 2, 3, 5-triphenylformazan (TPF) (Serra-Wittling et al. 1995). In brief, fresh soils (2.0 g dry weight (DW) soils) were incubated with 1 ml 3 % (*w*/*v*) TTC at 37 °C for 24 h. The dehydrogenase activity was expressed as milligram TPF per kilogram DW soil per 24 h, and the optical density of the filtered solution was determined at 485 nm.

Soil invertase activity was determined by the 3, 5-dinitrosalicylic acid method (Frankenberger and Johanson 1983). In brief, fresh soils (2.0 g DW soils) were incubated with 5 ml 0.17 M phosphate buffer (pH5.5) and 15 ml 8 % (*w*/*v*) sucrose solution at 37 °C for 24 h. The invertase activity was expressed as milligram glucose per gram DW soil per 24 h, and the optical density of the filtered solution was determined at 508 nm.

Soil neutral phosphomonoesterase activity was determined as described before (Wang et al. 2012). In brief, fresh soils (2.0 g DW soils) were incubated with 20 ml 0.1 M citrate–phosphate buffer (pH7.0) and 0.5 % (*w*/*v*) disodium phenyl phosphate at 37 °C for 24 h. The neutral phosphomonoesterase activity was expressed as milligram *p*-nitrophenol per gram DW soil per 24 h, and the optical density of the filtered solution was determined at 510 nm.

Soil urease activity was determined with the indophenol blue colorimetric method (Gosewinkel and Broadbent 1984). In brief, fresh soils (2.0 g DW soils) were incubated with 20 ml 0.96 M citrate buffer (pH6.7) and 10 ml 10 % urea at 37 °C for 24 h. The urease activity was expressed as milligram $NH_4^+$-N per gram DW soil per 24 $h^{-1}$, and the optical density of the filtered solution was determined at 578 nm.

### DNA extraction

In the next day of sampling, soil DNA were extracted from 0.5 g fresh soils using a Fast DNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA). The extracted soil DNA was dissolved in 70 μl Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH=8.0) and stored at −20 °C for further analysis.

### Real-time quantitative PCR of bacterial 16S rRNA genes

Using primer sets 519F/907R (519F: 5′-CAGCMGCCGCGGTAATWC-3′, 907R: 5′-CCGTCAATTCMTTTRAGTTT-3′), copy numbers of bacterial 16S rRNA gene fragments were quantified by the real-time quantitative PCR (qPCR), with a C1000$^{Tm}$ Thermal Cycler equipped with a CFX96$^{Tm}$ Real-Time system (Bio-Rad, CA, USA) (Biddle et al. 2008). Assays were set up by the SYBR *Premix ExTaq*$^{TM}$ *Kit* (TaKaRa, Dalian). Briefly, 25-μl reaction mixtures contained 12.5 μl of SYBR® *Premix ExTaq*$^{TM}$, 0.5 μM of each primer, and 1.0 μl template containing 2–9 ng DNA. Blank controls were run with water as the template instead of soil DNA extract. The PCR program was initially set up at 94 °C for 2 min, followed by 30 amplification cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The cycle threshold ($C_T$, PCR cycle numbers that reach the threshold fluorescence level) was set automatically by the system. To evaluate amplification specificity, melt curve analysis was performed at the end of the PCR run.

To generate a qPCR standard curve, a single clone containing the correct insert was grown in the Luria–Bertani medium, and the plasmid DNA was then extracted, purified, and quantified. A tenfold dilution series of the plasmid DNA was generated as standards covering seven orders of $10^3$ to $10^9$ copies of the template per assay. The standard curve was generated by plotting $C_T$ versus the copy number. Based on the curve, the copy numbers of bacterial 16S rRNA genes in the soil DNA extracts were calculated by extrapolating their $C_T$ values. All determinations of qPCR were performed in triplicates, and a range of 97.4–104 % amplification efficiencies ($E=10^{-1/\text{slope}}-1$, Rasmussen 2001) were obtained ($R^2$=0.976–0.997). The final quantities of bacterial 16S rRNA genes were calibrated against the total DNA concentrations in the extracted soil and the water control.

### PCR–denaturing gradient gel electrophoresis analysis

Soil bacterial community composition was characterized by the PCR-DGGE fingerprinting analysis with the primer set of 341F-GC/907R (341F-GC: 5′-CCCCCGCCGCGCGCGG CGGGCGGGGCGGGGG CACGGGCCGCCTACGGG AGGCAGCAG-3′, 907R: 5′-CCGTCAATTCMTTTRAGTTT-3′) (Muyzer et al. 1998). The PCR was programmed as follows: initial denaturation at 95 °C for 7 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min. For soil bacillus community structure, nested PCR was used (De Clerck et al. 2004) with the primer set pB/pH (pB: 5′-CGATGCGTAGCCGACCTGAG-3′, pH: 5′-AAGGAGGTGATCCAGCCGCA-3′) for the first round of PCR. The PCR profile consisted of an initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 60 s, annealing at 68 °C for 60 s, and extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. The product of this selective PCR was diluted 5,000-fold and used as the template for the second PCR, using primers Ec1055 and Ec1392 (Ec1055: 5′-ATGGCTGTCGTCAGCT-3′, Ec1392: 5′-ACGGGCGGTGTGT AC-3′). The PCR profile consisted of an initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 40 s, and

extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min.

The PCR products were separated on 6 % (*w*/*v*) polyacrylamide gels with a 30–70 % denaturing gradient (urea and formamide). DGGE gels were run at a constant voltage of 85 V for 16 h at 60 °C in 1× Tris–acetic–EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH8.3), stained in 1:10,000 SYBR® Green I, and subsequently scanned with Gel Doc™ EQ Imager combined with the Quantity One 4.4.0 (Bio-Rad, CA, USA).

The representative bands were excised, left overnight in 25 μl Milli-Q water, re-amplified, and run again on the DGGE system to ensure purity and correct mobility of the excised DGGE bands. Correct PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) before cloning.

### Cloning, sequencing, and phylogenetic analysis

The purified PCR amplicons of the excised DGGE bands were cloned into a pMD18-T vector (TaKaRa, Dalian) and transformed into *Escherichia coli* DH5α competent cells. Six random clones containing correct gene size for each DGGE band were sequenced by the Invitrogen Sequencing Department in Shanghai, China. The DNASTAR software package was used to manually check and compare the clone sequences. One representative clone sequence with high quality after the sequence comparison from each band was used for the phylogenetic analysis. Together with the top three BLAST hits of the homologous gene sequences, the DGGE band sequences were used to build a basic phylogenetic tree by the neighbor-joining method using the software package of Molecular Evolutionary Genetics Analysis version 4.0 (Tamura et al. 2007). The tree topology was further evaluated by different methods, including the minimum evolution and maximum parsimony. The phylogenetic relationships of target gene sequences to the closest homolog in the GenBank were then inferred. The GenBank accession numbers for bacterial and *Bacillus* 16S rRNA gene fragments sequenced in this study are AB705417 to AB705442.

### Statistical analysis

All results were expressed on an oven-dried soil weight basis (105 °C, 24 h). Mean separation was conducted based on Tukey's multiple range tests. Differences at $P<0.05$ were considered statistically significant. The PCR-DGGE fingerprints based on target gene banding patterns were photographed and digitized using the Quantity One software (Bio-Rad, CA, USA). Using the digital matrix obtained from DGGE, the similarities (or dissimilarities) among different treatments could be quantified using the cluster analysis. Euclidean distances were calculated from relative positions and intensities of bands, and the samples were clustered using the Pearson's product–moment coefficient and an Unweighted Pair Group Methods with Arithmetic mean algorithm.

## Results

### Yields and rebaudioside A production

Compared to the no-fertilization control, fertilizations (sole NPK, FS + NPK, and CS + NPK) all significantly increased yields of the *Stevia* plant ($P<0.05$, Table 1). The highest yield was observed for the soil treated with CS + NPK, followed by FS + NPK. The patterns of *Stevia* quality, characterized by rebaudioside A concentration, were similar to those of yields, the highest quality with CS + NPK compared to the control.

### Soil enzyme activities

Among the four tested soil enzyme activities, except neutral phosphomonoesterase, activities of dehydrogenase, urease,

**Table 1** Effects of different fertilization on yields of *Stevia* and rebaudioside A concentrations in *Stevia* leaf as well as four soil enzyme activities

| Treatments | Control | NPK | FS + NPK | CS + NPK |
|---|---|---|---|---|
| *Stevia* yields (t $ha^{-1}$) | 2.03 (0.11) c | 2.75 (0.08) b | 2.98 (0.18) ab | 3.08 (0.08) a |
| Rebaudioside A (g$kg^{-1}$) | 90.20 (3.80) a | 91.00 (0.50) a | 91.01 (4.70) a | 93.20 (2.10) a |
| Dehydrogenase (mg$kg^{-1}$) | 9.90 (1.80) c | 5.10 (1.20) c | 35.50 (17.30) b | 64.90 (4.20) a |
| Invertase (mg$g^{-1}$) | 8.94 (0.10) b | 7.65 (0.53) b | 14.50 (3.74) a | 14.28 (3.57) a |
| Phosphomonoesterase (mg$g^{-1}$) | 2.46 (0.20) a | 2.49 (0.01) a | 2.72 (0.30) a | 2.75 (0.93) a |
| Urease (mg$g^{-1}$) | 0.46 (0.04) b | 0.44 (0.05) b | 0.49 (0.07) ab | 0.56 (0.05) a |

Numbers in parentheses are standard deviations. Different letters in each row indicate significant differences ($P<0.05$)

*Control* no chemical fertilization and no *Stevia* residue returning control, *NPK* chemical N, P, and K fertilization, *FS + NPK* fresh *Stevia* residue plus NPK, *CS + NPK* composted *Stevia* residue plus NPK

and invertase were generally significantly increased under the addition of either fresh or composted *Stevia* residue ($P<0.05$) (Table 1), compared to both the no-fertilization control and the sole NPK fertilization. In contrast, activities of dehydrogenase, invertase, phosphomonoesterase, and urease were similar between the no-fertilization control and the sole NPK fertilization.

## Copy number of bacterial 16S rRNA genes

The copy numbers of bacterial 16S rRNA genes in soils varied from $3.1\times10^8$ to $6.5\times10^8$ $g^{-1}$ DW soil and were significantly increased under CS + NPK and FS + NPK ($P<0.05$, Fig. 1a). In contrast, copy numbers of bacterial 16S rRNA genes were similar between the no-fertilization control and the sole chemical NPK fertilization.

## Bacterial and *Bacillus* community structures

Fingerprinting profiles of PCR-DGGE were used to investigate effects of different fertilizations on soil bacterial community compositions (Fig. 1b), as well as that of the functional guild, *Bacillus* (Fig. 2a). Cluster analysis revealed that at 0.83 Euclidean distance (point A in Fig. 1c), bacterial compositions under three fertilizations, NPK, FS + NPK, and CS + NPK, were grouped into one cluster and separated from that under no-fertilization control. Among fertilization treatments, soil bacterial compositions were shifted to a greater extent under CS + NPK and FS + NPK than under the sole NPK (point B and C in Fig. 1c). With the greatest effect due to the addition of *Stevia* residues, the intensities of DGGE bands 1, 2, 3, 8, 14, and 18 were increased under CS + NPK and FS + NPK than

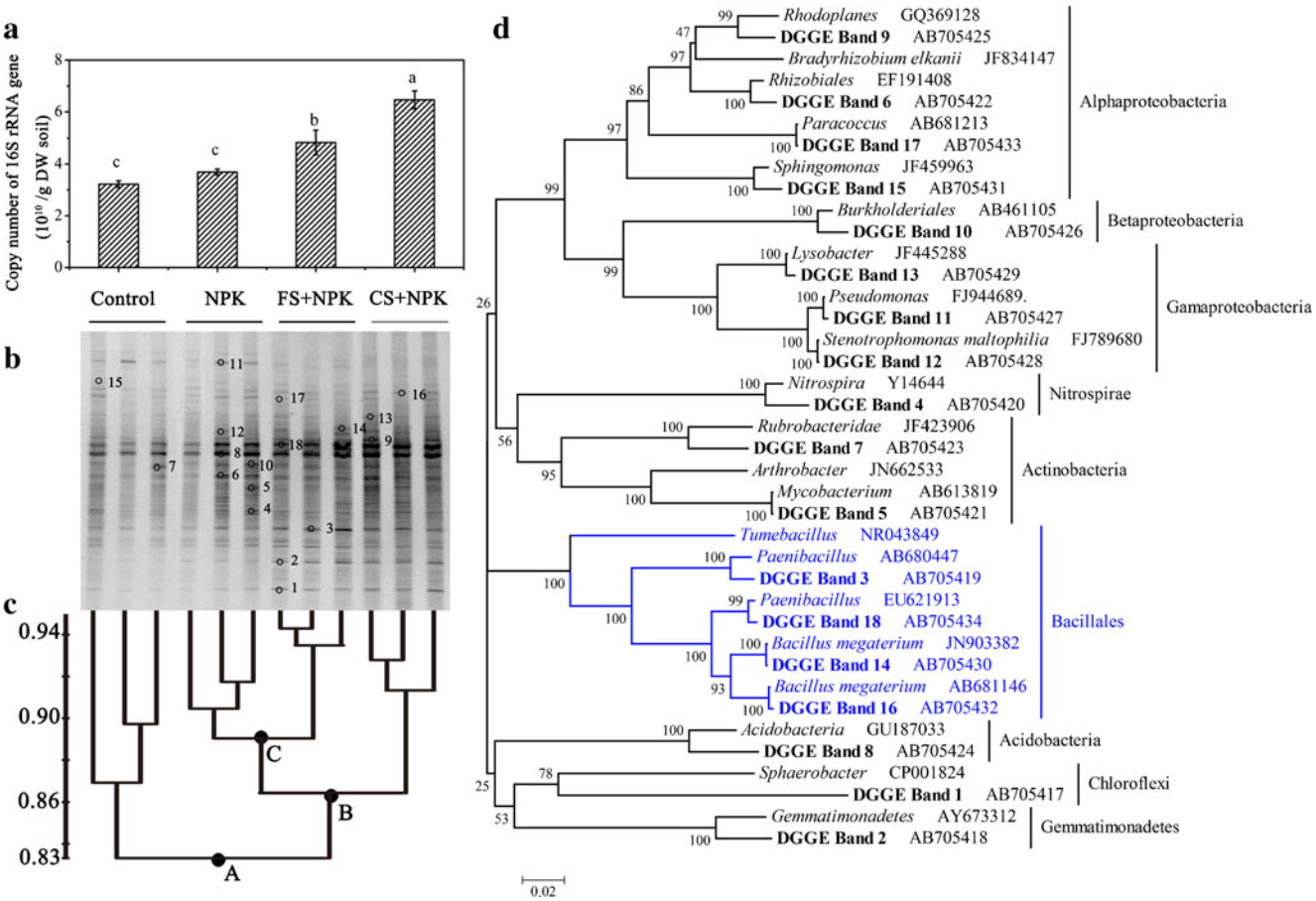


**Fig. 1** Soil bacterial community in different fertilization treatments. *Control* no chemical fertilization and no *Stevia* residue returning control; *NPK* chemical N, P, and K fertilization; *FS + NPK* fresh *Stevia* residue plus NPK; *CS + NPK* composted *Stevia* residue plus NPK. *a* The copy number of bacterial 16S rRNA gene in different treatments. Significant differences are indicated by *different letters* over *error bars* ($P<0.05$); *b* DGGE fingerprinting profiles of soil bacterial 16S rRNA genes. Sample designations are indicated above each DGGE lane. The bands excised for sequencing are circled and numbered from 1 to 18, respectively; *c* cluster analysis of DGGE fingerprint profiles of soil bacterial 16S rRNA genes; *d* phylogenetic tree based on soil bacterial 16S rRNA genes retrieved from the DGGE bands and its closest relatives deposited in GenBank. Bootstrap values based on 1,000 replicates are indicated by the *numbers* at the *nodes*. The *scale bar* indicates the number of nucleotide acid substitutions per site

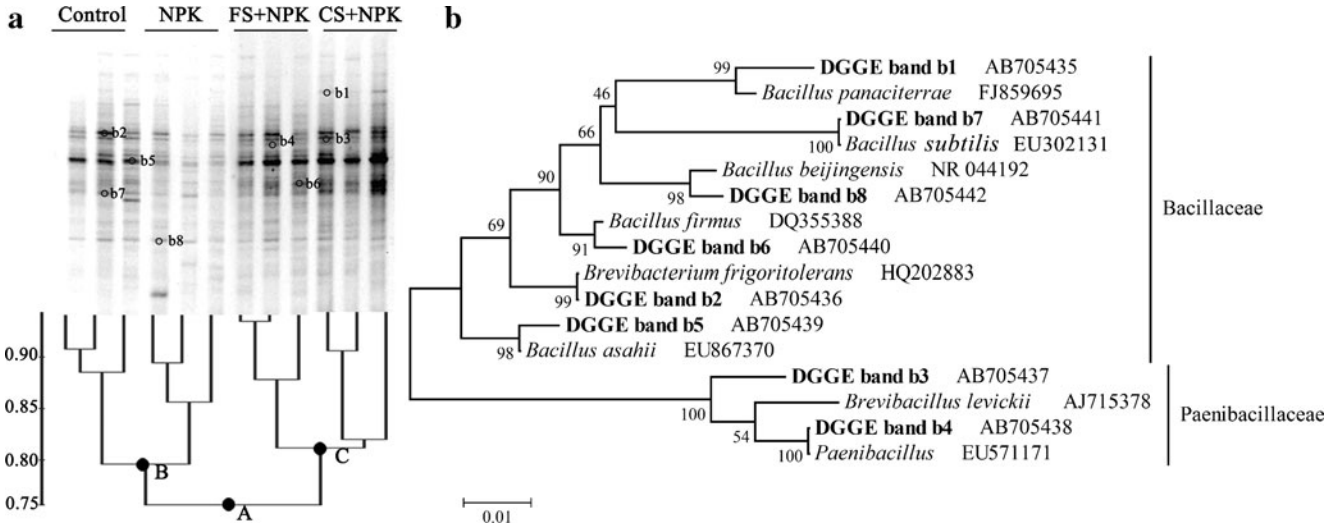


**Fig. 2** DGGE fingerprinting profiles of soil *Bacillus* 16S rRNA genes in different fertilization treatments. *Control* no chemical fertilization and no *Stevia* residue returning control; *NPK* chemical N, P, and K fertilization; *FS + NPK* fresh *Stevia* residue plus NPK; *CS + NPK* composted *Stevia* residue plus NPK. Sample designations are indicated above each DGGE lane. The bands of *Bacillus* excised for sequencing are circled and numbered from *b1* to *b8*, respectively

under the sole NPK and no-fertilization control. Phylogenetic analysis further revealed that the majority of these phylotypes (DGGE bands 3, 14, and 18) were highly affiliated to *Bacillales* (Fig. 1d).

Because the shift in soil bacterial composition could partly result from the stimulation of *Bacillus*, we further analyzed *Bacillus* community compositions under different fertilizations with functional primer sets (Fig. 2). Cluster analysis revealed that the *Stevia* residue returning treatments were grouped into one cluster at 0.75 Euclidean distance (point A in Fig. 2a), compared to those under no *Stevia* residue returning treatments of both sole NPK and no-fertilization control. Then the sole NPK group was separated from the no-fertilization control group at 0.80 Euclidean distance (point B in Fig. 2a). The pairwise comparison analysis of DGGE bands intensities indicated that both CS + NPK and FS + NPK did stimulate the growths of some band-related *Bacillus* in the sole NPK and no-fertilization control. For example, the average digitalized intensities of DGGE bands b1, b2, b5, b6, and b7 all increased. Phylogenetic analysis revealed that these phylotypes were all affiliated to *Bacillaceae* (Fig. 2b).

## Discussion

### *Stevia* residue returning increases crop yields and qualities

Returning of *Stevia* residue, either as fresh or composted organic matter, increased the yields and rebaudioside A of *S. rebaudiana*. As a side product of an increasing worldwide production of *S. rebaudiana* (Bertoni) to meet the ever-growing human demands, a large number of *Stevia* residues from such agricultural and industrial activities are expected. How to efficiently and effectively utilize these residues, while a sustainable soil productivity and fertility is conserved, becomes an eminent issue. Returning crop straw back to the field is one of the feasible means for such a purpose (Tirol-Padre et al. 2005). However, such a practice for the cash crop, *Stevia* has not been currently implemented. Therefore, this new study investigated the effects of two types of *Stevia* residue returning on its own growth and soil microbial characteristics. Both CS + NPK and FS + NPK significantly increased yields and qualities of *Stevia*, compared to those of the no-fertilization control (Table 1). Meanwhile, they even positively affected *Stevia* growth to a greater extent than sole NPK did due to the unique properties of *Stevia* leaf. *Stevia* leaf contains nutrients such as calcium, phosphorus, iron, and potassium (Savita et al. 2004). Even after sweetener extraction, the majority of nutrients are retained in the residues of the *Stevia* leaf, for example 21.9 $mg\,g^{-1}$ N in fresh and 42.0 $mg\,g^{-1}$ N in compost. These nutrients can promote plant growth. Meanwhile, small molecular weight organic acids, such as oxalate, a common fermented product of straw (Li et al. 2011), can chelate metal elements to increase their bioavailability for plants. As a consequence, the addition of *Stevia* residues was more effective than the sole NPK fertilization (Table 1).

### *Stevia* residue returning enhances soil enzyme activities

Besides the direct promotion, components of *Stevia* residues can stimulate growths of soil microorganisms and increase their activities, which can favor crop growth in a sustainable

way. Dehydrogenase activity has been considered as an indicator of the overall soil microbial activity, while urease, phosphomonoesterase, and invertase activities characterize soil microorganisms' ecological function involved in soil N, P, and C cycles, respectively. In this investigation, we found that *Stevia* residue amendments significantly increased their activities, except neutral phosphomonoesterase (Table 1). The stimulations of these soil enzyme activities may result from physiologically active compounds in *Stevia* leaves. As reported, the *Stevia* leaf contains up to 21 % protein and 35.2 % carbohydrates on dry weight basis (Tadhani and Subhash 2006), and almost all of the indispensable amino acids, including tyrosine and cysteine (Abou-Arab et al. 2010), which means that *Stevia* residues can be readily consumed by soil microbes. In contrast, other crop straws, which mainly consist of cellulose, hemicellulose, lignin, etc., are hard to be utilized by soil microbes. As a consequence, the returning of *Stevia* residues increased soil enzyme activities to a greater extent, which is characterized by higher dehydrogenase, urease, and invertase (Table 1). Interestingly, for neutral phosphomonoesterase activity, it did not show significant differences among all the treatments. The underlying mechanisms could be that neutral phosphomonoesterase activity was undergone by two contrasting effects: (1) the increase in the enzyme activity due to microbial growth and (2) the decrease in the enzyme activity due to the repression of the enzyme synthesis due to sufficient available P, especially under the application of inorganic P (Nannipieri et al. 2008). Specifically, the increase in its enzyme activity due to microbial stimulation could be counteracted by the decease due to the application of inorganic P. Besides, it should be noted that the measured enzyme activity is a potential and not real enzyme activity (Nannipieri et al. 2012). To corroborate the differentiations in soil enzyme activities and to prove our postulation, we further conducted a molecular analysis on soil bacterial community composition and abundance.

## *Stevia* residue returning enhances soil microbial genetic diversity

Field managements, such as tillage, cropping systems, and fertilization, affect soil microbial community composition (He et al. 2008; Chaparro et al. 2012) and enzyme activities (Table 1). *Stevia* residue returning did provide organic matter and nutrients for promoting soil microbial growth, and soil bacterial abundances were hence increased under both CS + NPK and FS + NPK (Fig. 1a). The qPCR results further corroborated the responses of soil enzyme activities to the addition of *Stevia* residues. Nutrients would be then released by such stimulated soil enzymes activities for an enhanced plant growth (Table 1). Concomitantly, soil bacterial community compositions were shifted under *Stevia* residue returning (Fig. 1c). Cluster analysis revealed that *Stevia* residue returning in both CS + NPK and FS + NPK brought greater shifts into the bacterial composition than the sole NPK (Fig. 1b). Phylogenetic identification revealed that there were at least several dominant bacterial phyla, such as alpha-, beta-, gamma-*Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes*, and *Bacillales*, present in soils (Fig. 1d). Among these phyla, the intensities of band-related *Bacillales* were increased (Fig. 1b), indicating that some *Bacillales* were stimulated by *Stevia* residue returning to a greater extent than other soil bacteria. *Bacillus* are one of the important functional guilds in agricultural soils, due to their ecological functions, such as the stimulation of nutrient uptake by host plants, antagonisms against plant pathogens and insect pests, and stimulation of plant host defense mechanisms (Seldin 2011). For such reasons, we further analyzed the community structure of *Bacillus* using their unique functional primer set. Namely, a greater positive influence on *Bacillus* community composition appeared under both CS + NPK and FS + NPK than under the sole NPK. Almost all band-related *Bacillus* were stimulated by *Stevia* residue returning, such as DGGE bands b1, b5, and b7. Soil *Bacillus* in *Stevia* residue-treated soils were phylogenetically identified as *Baciilaceae* and *Paenibacillaceae* (Fig. 2b). In fact, aerobic endospore-forming bacteria are ubiquitous in agricultural soils (Govindasamy et al. 2010), and they have great advantages over other plant growth-promoting rhizobacteria strains due to their stable maintenance in rhizosphere soil. Indeed, *Bacillus asahii* (DGGE band b5 in Fig. 2b) is stimulated by long-term organic fertilization (Chu et al. 2007), and *Bacillus subtilis* (DGGE band b7 in Fig. 2b) has been shown to be involved (Rudrappa et al. 2008). Both species are positively correlated with crop yields. Furthermore, the comparisons of *Bacillus* community composition between soils under CS + NPK and FS + NPK as well as the fresh *Stevia* residues revealed that the increase in *Bacillus* abundance was from the stimulation of indigenous bacteria, rather than from the introduction of composted *Stevia* residues (Fig. S1).

In conclusion, the returning of *Stevia* residues generally stimulated the growth of the plant itself and its specific compound, rebaudioside A. The addition of *Stevia* residues into soil also generally enhanced enzyme activities and the growth of soil microbes, especially those with important ecological behaviors; meanwhile, their microbial community compositions shifted. Therefore, the used *Stevia* residues can not only increase *Stevia* yield and quality by stimulating soil microbes, but also avoid pollution by waste discharge.

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